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Related cell-surface antigens expressed with positional specificity in *Drosophila* imaginal discs

(*Drosophila* development/glycoproteins/monoclonal antibodies)

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ABSTRACT **We have isolated three classes of monoclonal antibodies against *Drosophila* cell-surface antigens that are expressed with positional specificity in imaginal discs. Comparison of immunofluorescence patterns with the wing-disc fate map reveals that expression of the antigens is not directly related to the specific type of cuticular structure that a cell will make upon differentiation but depends on the position of the cell in the undifferentiated disc epithelium. On mature wing discs, each class of position-specific (PS) antibody binds nonuniformly with respect to the dorso-ventral compartment boundary, with PS1 antibodies binding primarily to dorsal cells and PS2 antibodies, to ventral cells. Antibodies of the different PS classes extract similar but nonidentical sets of large glycoproteins from cell lysates, and antibodies of the most general class, PS3, recognize the PS1 and PS2 antigens in addition to PS3-specific components. Thus, the distributions and molecular characteristics of the PS antigens suggest that the molecules are structurally and functionally related to one another.**

Much of the adult *Drosophila* epidermis and some internal structures derive, at metamorphosis, from imaginal discs, which are present in larvae as convoluted inpocketings from the larval hypoderm. [Imaginal disc morphology and development are reviewed by Poodry (1).] In mature third-instar larvae each imaginal disc, although still largely undifferentiated, is stably determined to make a particular region of the adult (2, 3), and the cells within a particular part of a disc are specified to make a particular adult structure (4) (see Fig. 1). This precise specification of pattern within the disc appears to depend on intercellular interactions (6, 7). Clonal analysis has shown that discs are subdivided into compartments, defined by the observation that clones of genetically marked cells do not cross a compartment boundary into the territory of neighboring compartments (8). Genetic studies indicate that at least some of these compartments are units of gene action in the development of the epidermis (9, 10), although the relationship between compartments and the precise specification of pattern elements is not clear.

We have studied the molecular basis of early disc development by using monoclonal antibodies to detect cell-surface antigens with heterogeneous distributions in larval discs before overt differentiation occurs. We previously described (5) the distribution of one such antigen, recognized by monoclonal antibody DK.1A4. Comparison of this antigen's distribution with a cell-lineage analysis of the mature wing imaginal disc (which makes the adult wing and associated mesothorax) showed that expression of the antigen correlated with the dorso-ventral lineage restriction (the border between dorsal and ventral compartments) in the epithelium. It was also clear that the expression of the antigen was not tightly linked to the type of adult structure that a disc cell was specified to differentiate, but seemed rather to depend on the position of the cell within the disc epithelium. We have now isolated numerous monoclonal antibodies displaying two related positional specificities, along with more antibodies showing the original DK.1A4 specificity. Here we describe these specificities in mature wing discs and show that the antigens recognized by these antibodies are related structurally as well as by distribution.

MATERIALS AND METHODS

Production of Antibodies. The procedure used for the generation of all position-specific (PS) hybridomas was similar to that used to obtain the clone producing PS1 antibody DK.1A4 (5). To produce the other PS antibodies, BALB/c mice were immunized with sonicated imaginal discs (antibodies prefixed CF) or purified antigens extracted by DK.1A4 (antibodies prefixed DT). For CF, material diluted in *Drosophila* Ringer's solution was injected i.p. 5 days before an i.v. booster injection of similarly diluted material, which was given 4 days before splenectomy and fusion. For DT, i.p. and s.c. injections (in complete Freund's adjuvant) on day 0 and an i.p. injection (in complete Freund's adjuvant) on day 20 preceded an i.v. booster injection (in phosphate-buffered saline) on day 36; splenectomy and fusion were done on day 40. Hybridomas were screened by immunofluorescence using whole imaginal discs, and positive lines were cloned by limiting dilution. For the production of antibody, the hybridomas were grown as ascites cells in mice. The ascites fluid was either diluted directly (most fluorescence experiments) or the antibodies, all of which were IgGs, were purified by affinity chromatography on protein A-Sepharose CL-4B (Pharmacia) (most affinity-isolation experiments). The antibodies primarily used in this study were CF.5E5 and DK.1A4 (PS1 class), CF.2C7 (PS2 class), and CF.6G11 and DT.2H10 (PS3 class).

Immunofluorescence. Imaginal discs were dissected from larvae and incubated in antibody solutions at room temperature. Incubations in primary antibodies were generally for 45 min at dilutions of 100–1000, and secondary antibody incubations were for 30 min at dilutions of 60–200, all with frequent stirring. The first antibody was typically diluted in a nitro blue tetrazolium staining solution containing metabolic poisons and Tris buffer (11). This has two important effects: (*i*) The disc is stained dark blue—this opacity greatly reduces background fluorescence. (*ii*) The tissue appears to be mildly fixed, thus inhibiting antibody-induced reorganizations of antigens on the plasma membrane. Washes (15–30 min) and subsequent antibody dilutions were in RPMI 1640

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Abbreviations: PS, position-specific; gp, glycoprotein.

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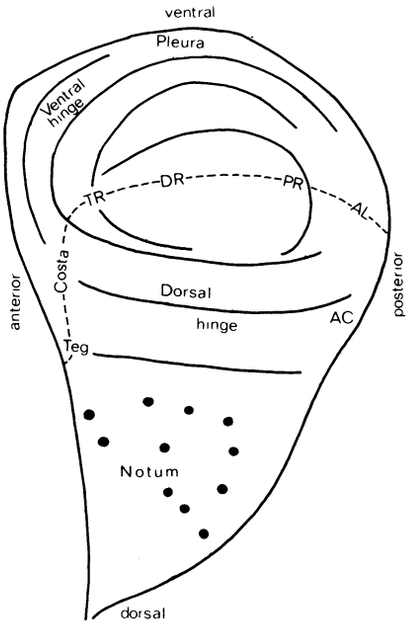

FIG. 1. Wing imaginal disc showing the fate-map locations of the adult structures derived from the disc [after Bryant (4)]. The diagram shows the columnar epithelial face of the disc, which gives rise to the majority of the adult structures. Solid lines indicate folds in this layer. The various regions (dorsal, ventral, anterior, posterior) are defined by the final position, in the fly, of the structures derived from these regions. This orientation is preserved in all subsequent figures. The disc everts prior to its final differentiation, so that peripheral disc tissue gives rise to proximal adult thoracic structures (notum and pleura) while the central area generates the more distal wing blade structures. Double-row (DR) bristles, for example, are found at the tip of the wing. Other abbreviations: TR, triple-row bristles; PR, posterior-row hairs; AL, alar lobe; Teg, tegula; AC, axillary cord. Filled circles show the positions of major notal bristles. The dotted line represents the position of the dorso-ventral lineage restriction (5). The disc is actually a flattened sac of cells; the reverse face is composed largely of a smooth squamous epithelium called the peripodial membrane.

medium (Flow Laboratories) with 5–10% bovine serum, except that incubations or washes following formaldehyde fixation were in 50 mM phosphate buffer/150 mM NaCl, pH 7.0 containing 0.2–0.5% serum or bovine serum albumin. Secondary antibodies were either fluorescein-conjugated goat anti-mouse IgG (Antibodies Inc.) or tetramethylrhodamine-conjugated rabbit $F(ab')_2$ anti-mouse IgG (a gift of R. J. Morris). Typically, discs were labeled with a primary PS monoclonal antibody, then with a secondary antibody, and then fixed in 2% formaldehyde for 10 min. This was followed by an incubation with a monoclonal antibody that binds to all of the epithelial cells (either DA.1B6 or CF.7A6, which show similar specificities; see ref. 12) and then an incubation with another secondary antibody conjugated to a different fluorochrome. This procedure allows the simultaneous visualization of both the PS antigen distribution and the detailed morphology of the disc and also controls for possible permeability artifacts. For double-labeling with different PS antibodies, the second monoclonal antibody was conjugated directly to fluorescein isothiocyanate and the formaldehyde fixation step was done last.

Discs were mounted in 70% glycerol/30% 0.1 M Tris Cl, pH 9, to which was added 0.2%–5% *n*-propyl gallate (13), and viewed under epi-illumination on a Zeiss photomicroscope. Photographs were on Kodak Tri-X film developed with Diafine developer.

Affinity Purification Procedures. Discs [hand-dissected or mass-isolated by a modification of the procedure of Eugene and Fristrom (14)] or cells of the Emal[1] *Drosophila* cell line were lysed by the addition of an equal volume of double-strength lysis buffer [lysis buffer is 20 mM Tris Cl/150 mM NaCl/1 mM $MgCl_2$/1 mM $CaCl_2$/0.5% Nonidet P-40 (BDH)/0.5% crystalline bovine serum albumin/0.1% Trasylol (Calbiochem)/1 mM phenylmethylsulfonyl fluoride (Sigma)/0.1% NaN_3, pH 8.1]. Discs required disruption by brief sonication. The lysate was kept at 0°C for 30 min, to solubilize membrane components, and then centrifuged at 12,000 × *g* for 3 min at 4°C. The supernatant was preincubated at 4°C for 3–10 hr with deactivated Affi-Gel 10 (Bio-Rad), to remove material that bound Affi-Gel nonspecifically. Following this preincubation, a portion of the supernatant, typically 0.1 ml, was added to 40 μl of pelleted Affi-Gel 10 to which specific PS antibodies had been covalently attached (Bio-Rad bulletin 1085) at a concentration of 2 mg of antibody/ml of Affi-Gel. The mixture was gently agitated for 4 hr at 4°C. The Affi-Gel–antibody–antigen was collected by

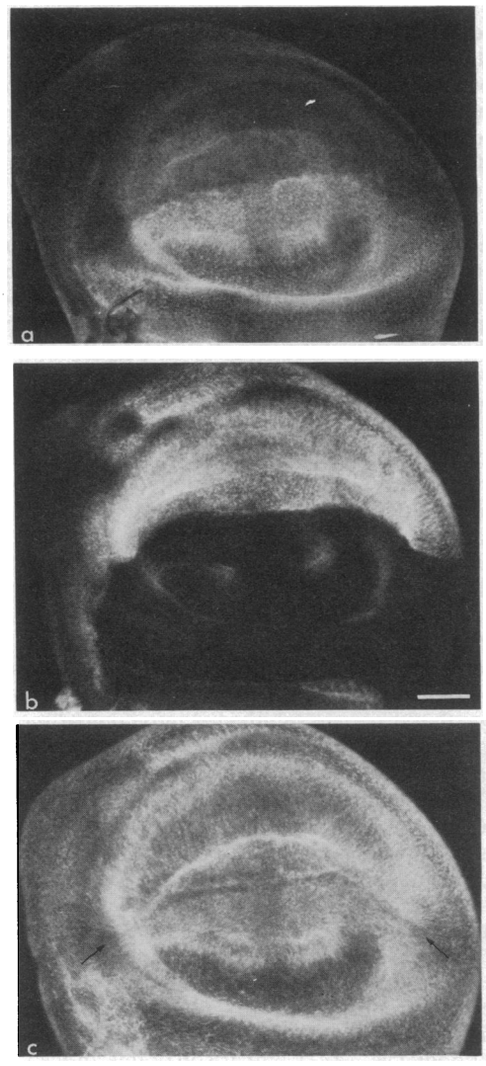

FIG. 2. Immunofluorescence labeling of late third-instar wing imaginal discs with PS antibodies. (*a*) Disc labeled with a PS1 antibody (see also ref. 5). The fluorescence is much more intense in the dorsal region of the disc. (*b*) Disc labeled with a PS2 antibody. Most of the ventral half of the disc is intensely labeled, although there are ventral regions, particularly in the anterior (left side of figure), that show less fluorescence. While most of the dorsal half is completely negative, there are a few central areas that show low levels of fluorescence. (*c*) Disc labeled with a PS3 antibody. Both dorsal and ventral regions are labeled, although there is less fluorescence in anterior or ventral regions (compare with *b*) and along a line separating the dorsal and ventral halves of the disc (arrows). (Bar = 50 μm.)

centrifugation, washed twice with 10 ml of cold wash buffer (lysis buffer without bovine serum albumin and containing Nonidet P-40 at 0.2%) and once with 10 ml of 5 mM Tris Cl, pH 8.1. The Affi-Gel–antibody–antigen was then spun dry in a yellow Eppendorf pipet tip plugged with glass wool, and antigens were eluted with 60 μl of gel electrophoresis sample buffer (125 mM Tris Cl/2% $NaDodSO_4$/20% glycerol, pH 6.5). After the addition of 0.1 M dithiothreitol, eluates were analyzed by electrophoresis on polyacrylamide gels, using the gel system of Laemmli (15). Figs. 4 and 5 show antibody–Affi-Gel extracts. We have also isolated PS antigens using protein A-Sepharose to bind the antigen–antibody complex. Following centrifugation of the disc lysate, the supernatant was preincubated with protein A-Sepharose for 30 min at 4°C. Aliquots of lysate were then incubated for 1 hr at room temperature with antibody. This solution was then mixed with 40 μl of protein A-Sepharose/lysis buffer (1:1), and gently agitated at 4°C for 30 min. The antigen–antibody–protein A-Sepharose was collected by centrifugation and washed three times with each of the following: (*i*) 20 mM Tris Cl, pH 8.1/150 mM NaCl/1 mM $MgCl_2$/0.2% Nonidet P-40, (*ii*) 10 mM Tris Cl, pH 7.4/500 mM LiCl/0.1% $NaDodSO_4$/0.2% Nonidet P-40, and (*iii*) 10 mM Tris Cl, pH 7.4. The washed beads were suspended in electrophoresis sample buffer to extract the antigens and antibodies.

Visualization of Gel Bands. After electrophoresis and transfer to nitrocellulose unlabeled antigens were visualized by the procedure of Hawkes (16), except that fetal calf serum was replaced by polyvinylpyrrolidone-360. This procedure involves incubating the blot with concanavalin A, which binds to all of the antigens, and subsequently with horseradish peroxidase (Sigma grade VI), which binds the concanavalin A and can be localized histochemically. PS antigens were radiolabeled by surface-iodination of discs (by a procedure modified from ref. 17) and visualized by enhanced autoradiography of the gel (18), or labeled by incubating discs in [^{35}S]methionine (19) and visualized by gel fluorography (20).

RESULTS

Immunofluorescence Patterns of Position-Specific Antibodies on Wing Imaginal Discs. We have identified a number of monoclonal antibodies against three related categories of cell-surface antigens. All of the antibodies in these groups show differential binding to cells in different regions of imaginal discs, and we have designated the antibody categories PS1, PS2, and PS3. We have studied several antibodies of each specificity. The correspondence between imaginal disc fluorescence pattern and purified antigen bands described for the different PS categories has been found for at least five independently isolated antibodies both of the PS1 group and of the PS3 group; only one PS2 antibody has been used in affinity-purification experiments.

We previously described the immunofluorescence pattern of one PS cell-surface antibody (5). This pattern is characteristic of the group we now call PS1 antibodies. These antibodies bind predominantly to cells of the dorsal region of late third-instar wing discs (Fig. 2*a*). The shape and position of the dorso-ventral line defined by PS1 antibodies correlates with the line defined by the dorso-ventral lineage restriction in the disc (5). In contrast, PS2 antibodies show a complementary pattern, binding preferentially to ventral regions of mature wing discs (Fig. 2*b*).

While the PS1 and PS2 immunofluorescence patterns appear to define the dorso-ventral compartment border from opposite sides, neither antibody is completely specific for cells of one compartment. Some ventral binding of PS1 antibodies is detectable in mature wing discs, and PS2 antibodies bind to some dorsal regions. Moreover, PS2 antibodies do not bind to all of the ventral cells. There is less binding to a number of areas in the anterior ventral quadrant; this is more pronounced in discs from larvae close to pupariation, when little or no PS2 binding can be detected in a number of ventral areas.

PS3 antibodies bind to virtually the entire disc (Fig. 2*c*). Less intense fluorescence is observed in a number of regions, however, including a strip of cells along the dorso-ventral groove in the wing pouch (21) and a region in the anterior ventral quadrant of the disc. Indeed, double-labeling experiments indicate that the pattern of binding of PS3 antibodies is qualitatively similar to the sum of the PS1 and PS2 patterns.

Cross-linking of the PS antibody–antigen complexes to one another with anti-mouse IgG antibodies produces surface aggregates (Fig. 3 *a* and *b*), showing that the PS antigens are laterally mobile on the cell surface. All three categories of PS antibodies bind to the basolateral surfaces of the disc epithelial cells but do not bind to the apical (luminal) surface of the epithelium. This can be demonstrated either by stripping away the peripodial membrane cells on the reverse side of the disc, thus exposing the apical surface of the columnar

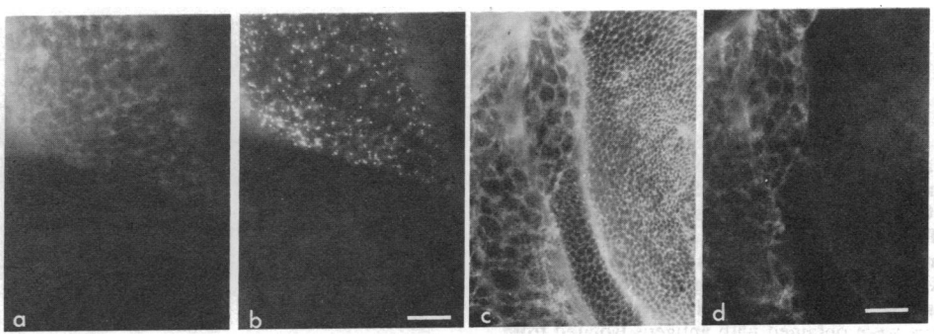

FIG. 3. (*a* and *b*) Antibody-induced lateral redistribution of PS antigens on the cell surface. Shown here are regions of the posterior margin from two discs labeled with a PS2 antibody. In *a*, the disc was fixed using our standard procedure; in *b*, the entire indirect immunofluorescence procedure was carried out under conditions in which the cells remained alive at room temperature (all the antibodies were diluted in RPMI 1640 medium), leading to the aggregation of the antigen–antibody complexes in distinct dots. This antibody-induced redistribution is seen for PS1 and PS3 antigens as well. Note that there is no detectable antibody binding to the dorsal part of the disc (lower half of the figure). (Bar = 10 μm.) (*c* and *d*) PS antibodies do not bind to the apical surface of the wing-disc epithelium. Before staining, part of the peripodial membrane over the wing pouch was removed, revealing the apical (luminal) surface of the columnar epithelium. (The disc here is viewed from the opposite direction to that shown in the previous figures.) In *c*, the binding of a non-PS antibody that binds to all surfaces of the epithelial cells (see *Materials and Methods*) shows the basal outlines of the squamous peripodial membrane cells and the smaller apical profiles of the underlying columnar wing pouch cells. In *d*, the PS3 immunofluorescence for the same field is shown. There is no detectable binding to the apical surface of the epithelium; only the basal outlines of the peripodial membrane cells are visible. This result also holds for PS1 and PS2 antibodies. (Bar = 25 μm.)

cells to the antibodies (Fig. 3 *c–d*), or by staining disc sections (unpublished data).

Biochemical Identification of the Position-Specific Antigens. We extracted the different PS antigens using affinity isolation procedures and compared the resulting extracts by NaDodSO$_4$/PAGE. The gels shown in this paper are of antigens extracted from the Emal[1] *Drosophila* cell line, which provides a convenient source of PS antigens. Similar results have been obtained with isolated imaginal discs.

All of the PS antigen extracts yield a complex set of gel bands. The isolated molecules can be visualized by a variety of techniques, including metabolic labeling with [^{35}S]methionine and surface-labeling of cells with ^{125}I. The antigen bands shown here were visualized by blotting gels onto nitrocellulose paper and probing the blots with concanavalin A and horseradish peroxidase. This method is faster and easier than the radiolabeling procedures, and it generally gives less nonspecific background. The different techniques give qualitatively similar results, showing that the antigens are all cell-surface glycoproteins (gp). Each antigen extract yields gel bands with apparent molecular masses of 92–125 kDa (gp92–gp125); the exact pattern of bands depends on which category of antibody is used (Fig. 4). Extraction with PS1 or PS2 antibodies produces a prominent band at ≈110 kDa (in some gels this broad band is resolved into two components; see Fig. 5) and two or more bands, depending on the antibody, at 116–125 kDa. The PS3 antibodies appear to extract all of these components, with an additional PS3-specific band, sometimes resolved as a doublet, at ≈92 kDa. None of the bands are extracted in the absence of a PS antibody or by our antibodies against other *Drosophila* cell-surface antigens. Since the bands represent glycoproteins, the apparent molecular weights from NaDodSO$_4$ gels can be used only as approximate indicators of size (22); we have used averages from different gel systems (15, 23) to generate the molecular mass values given here. Although the relative band intensities vary somewhat between experiments, the following features of the gel patterns are invariant: (*i*) there is a major band at ≈110 kDa for all three PS classes; (*ii*) there are two or more bands between 116 and 125 kDa, depending on the antibody class used; (*iii*) we have never observed gp125 by using a PS1 antibody; and (*iv*) we have never observed gp92 by using a PS1 or PS2 antibody.

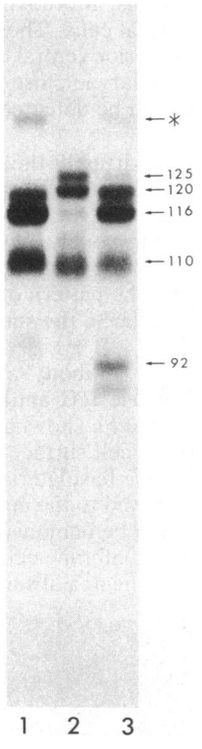

FIG. 4. Concanavalin A–horseradish peroxidase binding to nitrocellulose blots of immunoaffinity-purified PS antigens fractionated by NaDodSO$_4$/PAGE on 8% gels. Numbers at the bottom of the lanes correspond to PS categories. Numbers to the right indicate apparent molecular mass, in kDa. The various molecules extracted by the PS antibodies are glycoproteins, as judged by their ability to bind the lectin; this binding is completely inhibited by α-methylmannoside. Similar patterns were obtained with antigens isolated from cells that had been labeled by incubation with [^{35}S]methionine and from cells that had been surface-labeled with ^{125}I. Although the relative intensities of the bands may vary, gp110 is common to all three classes. The PS1 and PS2 antibodies extract class-specific sets of components between 116 and 125 kDa; the PS3 antibodies appear to extract all of these plus the gp92 doublet. In addition to these glycoproteins, we sometimes see one or more bands of lower mobilities in the antigen isolates (seen most clearly in lane 1, indicated by the asterisk to the right). Although apparently PS-antibody-specific, these high molecular weight bands are not always observed and may represent artifacts of our preparative procedures. The antigens shown on this blot were purified from cultured cells of the Emal[1] line; similar results were obtained with imaginal discs.

Thus, all three categories of antibody extract what appear to be similar sets of surface glycoproteins. In particular, the PS3 set includes, in addition to the unique gp92, components with mobilities similar to those of all the PS1 and PS2 glycoproteins. This, combined with the observation that the immunofluorescence pattern of PS3 antibodies was qualitatively similar to the sum of the PS1 and PS2 patterns, led us to believe that the PS1 and PS2 antigens might be recognized by PS3 antibodies. This was shown to be correct in experiments in which cell lysates were sequentially extracted with antibodies of the different classes (Fig. 5).

Prior extraction with any of the PS3 antibodies leaves no antigen extractable by either PS1 or PS2 antibodies (Fig. 5, lanes a and b). In contrast to this, PS1 and PS2 antibodies extract discrete subsets of the PS3 glycoprotein set. Thus, exhaustive extraction with PS1 antibody (lanes c and d; one extraction is usually sufficient) leaves the PS2 components intact for subsequent extraction by a PS2 antibody (lane e). Similarly, a PS2 antibody extracts all of the multi-component PS2 antigen without depleting the PS1 antigen (lanes f–h). Exhaustive prior extraction of a cell lysate with PS1 and PS2 antibodies leaves behind all of the doublet gp92, much of gp110, and some gp116 as residual PS3 antigen components

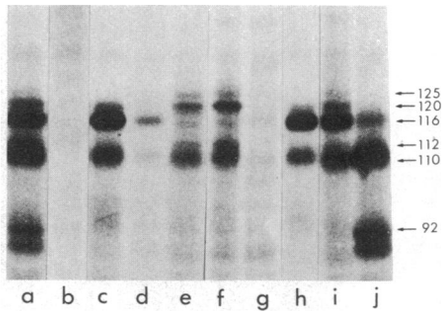

FIG. 5. Sequential extraction of cell lysates with different PS antibodies. Lanes a and b: Initial extraction by PS3 antibody (a), leaving no antigens extractable by PS1 and PS2 antibodies (b). Lanes c–e: Sequential extractions by PS1 antibody (c and d), followed by PS2 antibody extraction (e). There is no obvious PS2 depletion by the PS1 extraction. Lanes f–h: Sequential extractions by PS2 antibody (f and g) followed by PS1 extraction (h). There is no obvious PS1 depletion. Lanes i and j: Initial extraction by both PS1 and PS2 antibodies (i) followed by PS3 extraction (j). Only the "residual PS3 antigen" remains (see text). Extracts were fractionated by NaDodSO$_4$/PAGE on 8% gels, blotted onto nitrocellulose, and stained with concanavalin A–horseradish peroxidase. Numbers to the right are apparent molecular masses, in kDa.

(lanes i and j). The occurrence of residual PS3 antigen components, including the unique gp92, indicates that the PS3 antigen is not merely the sum of the PS1 and PS2 antigens. The distribution of this residual antigen (or antigens) in the imaginal wing disc is unknown, as we have no "residual PS3-specific" antibodies.

Another indication that the PS antigens are structurally related is that immunization of mice with antigens purified using one class of PS antibody can lead to the production of monoclonal antibodies showing another PS specificity. In two fusions, PS3 antibodies have been produced using PS1 antigens and vice versa. So far, no PS2 antibodies have been generated in this way.

The simplest interpretation of the above data is that the PS antigens are oligomeric complexes of the different glycoproteins resolved on our gels. It is more difficult to imagine that each of the glycoproteins carries the specific determinants recognized by the PS1 or PS2 antibodies, as well as the general PS3 determinant. A reasonable hypothesis is that PS3 antibodies recognize a common component (e.g., gp110–112) of a group of similar but nonidentical protein complexes, while the PS1 and PS2 antibodies recognize components that are specific to subsets of the PS group (e.g., gp116–125). A detailed biochemical analysis of the antigens, to be presented elsewhere, provides strong support for this hypothesis, and also indicates that the antigen bands may be placed into different subsets, based on similarities in their polypeptides (e.g., gp116–125, gp110–112, and the gp92 doublet).

DISCUSSION

The overt differentiation of a disc cell does not occur until metamorphosis, when the adult cuticle is deposited. Before this, the disc cells appear quite similar in general morphology (24), and the specification of adult pattern elements within the disc can be altered. Studies of major disc proteins by two-dimensional gel electrophoresis indicate few differences either between different discs or from region to region within a particular disc (25). Thus, until metamorphosis, the disc cells can be considered largely undifferentiated. We have shown here, however, that different regions of the wing imaginal disc do express different molecules (see also ref. 26). Antibodies of the PS1 and PS2 classes show a predominantly complementary binding pattern with respect to the border separating the dorsal and ventral compartments in the mature wing-disc epithelium. Also, each antibody class can be used to extract a related but nonidentical set of glycoproteins. The antigens recognized by all of the antibodies are found on the basolateral surface of the disc epithelium but not on the apical surface. These data together suggest that the three classes of antigens we have identified are functionally, as well as structurally, related to one another.

A first step in the determination of this function is the correlation of PS-antigen distribution with what is known of the processes of disc development. Comparison of the immunofluorescence patterns with the disc fate map shows that antigen expression does not relate to the type of cuticular structure that a particular disc region is specified to make (e.g., wing blade, hinge, or notum). For example, regions showing major differences in the expression of the PS1 and PS2 antigens will make identical wing-blade structures at metamorphosis, the only difference being that cells of one region will make the dorsal wing-blade surface and cells of the other, the ventral surface. Rather, antigen distributions seem to correlate with other events of disc organization, most clearly with dorso-ventral compartmentalization. But even here the correlation is not exact. For example, the PS2 immunofluorescence pattern in the mature wing disc shows large dark areas in the anterior ventral part of the disc and some significant fluorescent patches in regions which will become dorsal wing structures (Fig. 2*b*). An extensive developmental analysis, to be published elsewhere, shows that the spatial patterns of PS-antigen expression are dynamic during disc development and that discontinuities in PS-antigen expression in the disc epithelia generally correlate with morphogenetic events (e.g., the invagination of the wing pouch or the morphogenetic furrow of the eye). Furthermore, the PS antibodies bind to many embryonic, larval, and adult tissues, including those derived from germ layers other than ectoderm. These findings lead us to suggest that the PS antigens may be involved generally in cell recognition or adhesion phenomena during *Drosophila* development.

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